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Recombinant Nematode Nicotinic Receptor and Uses

Introduction

The nematode *C. elegans* has permitted identification and functional analysis of novel genes expressed in the nervous system (Bargmann, 1998). This invertebrate animal provides a highly effective genetic model with which to analyse *in vivo* molecules involved in chemical synaptic transmission (Jorgensen and Nonet 1995; Rand and Nonet 1997; Sattelle 1998). Neuromuscular cholinergic synapses in *C. elegans* have been analysed in detail stimulated by the finding that two major postsynaptic components, acetylcholinesterase (AChE, EC 3.1.1.7) and the nicotinic acetylcholine receptor (nAChR), are targets for widely used anthelmintic drugs. The hydrolytic enzymes AChEs which terminate the actions of ACh are inhibited by carbamates and organophosphates (Massoulié et al 1993). The nAChRs mediate the fast actions of the neurotransmitter ACh. When ACh binds to an nAChR molecule, the receptor molecule becomes transiently permeable to cations (Na^+, K^+, Ca^{2+}). Anthelmintic drugs such as levamisole, pyrantel and morantel are agonists and open channel blockers at native nematode muscle nAChRs (Martin 1996). The studies to date of cholinergic anthelmintic actions on recombinant ACR-16 (=Ce21) homomeric (probably neuronal) nAChRs (Ballivet et al; Raymond et al 1999) do not mimic the actions observed on native nematode muscle nAChRs.

Five polypeptide subunits surround a central ion channel in each nAChR molecule, each polypeptide having four transmembrane regions (M1-4) and a large N-terminal extracellular domain containing residues that form the ACh binding sites (Karlin, 1993; Unwin 1993; Lena & Changeux 1998). These subunits are classified as either α subunits, possessing two adjacent cysteines in loop C of the ACh binding site or non-α subunits, with no such adjacent cysteine motif.

Radioligand binding studies suggest the possibility of a diversity of nAChRs in *C. elegans*. For example, using [^{3}H]*meta*-amino levamisole, a high saturable affinity binding activity has been observed and is regulated in the course of development, the highest binding activity being detected in larval stages (Lewis et

al. 1980). Several approaches have been undertaken to characterize further nAChR diversity. For example it is possible to isolate mutants obtained by levamisole drug - resistance selection. The major levamisole resistance loci so far isolated, are as follows: *lev-1*, *lev-8*, *lev-9*, *lev-10*, *lev-11*, *unc-22*, *unc-29*, *unc-38*, *unc-50*, *unc-63* and *unc-74*. The *unc-50* gene encodes a product that may possibly be involved in assembly or transcriptional control of receptor units (Lewis et al 1987; Rand and Nonet 1997). The *lev-11* and *unc-22* genes are involved in muscle contraction, encoding respectively tropomyosin and twitchin (Williams and Waterston 1994, Beniam et al. 1989). Among these 11 resistance loci it has been shown that *lev-1* and *unc-29* both encode non-α subunit (Fleming et al. 1997), whereas *unc-38* encodes an α subunit. Molecular characterization of other loci remains to be done.

A separate genetic screen identified aldicarb resistant mutants. Aldicarb is an AChE inhibitor. This screen resulted in the isolation of 18 new loci, called *ric* genes (***r***esistant to ***i***nhibitor of ***c***holinesterase) and identified molecular components of both pre-synaptic and post-synaptic terminals (Nguyen et al. 1995, Miller et al. 1996). Of these loci, *ric-3* appears to be a good candidate for being a new nAChR subunit.

A genetic approach that screens for reduced pharyngeal pumping has identified other mutants and has resulted in the isolation of two interacting loci (*eat-2* and *eat-18*) which also encode candidate nAChR subunits (Raizen et al. 1995). A further strategy has employed cross - hybridization with either *Drosophila* nAChR cDNA or previously cloned *C. elegans* nAChR cDNA. Such techniques have permitted the cloning of three new nAChR subunits *acr-2*, *acr-3* and *acr-16* (Squire et al. 1995, Baylis et al. 1997, Ballivet et al. 1996).

Treinen *et al* (1998) discloses two functionally dependent acetylcholine subunits (*des-2* and *deg-3*) which are encoded in a single *C.elegans* operon. Their linkage in a single operon allows their coordinated and stoichiometric production in the same cells at the same time. The nAChR subunits DES-2 and DEG-3 are able to form a heteromeric nAChR composed of two different α subunits.

Finally, analysis of the recently (December 1988) completed *C. elegans* genome sequence shows the presence of 18 new α subunits and 2 new non-α subunits. There is therefore a large family of nAChR subunit genes in *C. elegans* for which the function remains to be elucidated.

The present invention relates to the cloning, by means of a cross-hybridization approach, of a new α nAChR subunit. We have shown that this new subunit is the product of *unc-63*, a levamisole - resistant gene. The amino acid sequence of the α nAChR subunit encoded by *unc-63* is shown in SEQ ID NO: 1. The present invention further encompasses analogs, variants, mutant forms, derivatives, and fragments of the UNC-63 α nAChR subunit, provided that such analogs, variants, mutant forms, derivatives, and fragments preserve at least one function of the wild-type UNC-63 α nAChR subunit as described herein. UNC-63 is expressed in body wall muscles of *C. elegans* and in certain motor neurons. This subunit has been co-expressed with UNC-29 and LEV-1 in *Xenopus* oocytes resulting in the first robust heterologous expression of an invertebrate recombinant heteromeric nAChR. The finding that this expressed nAChR containing UNC-63 mimics several of the properties of native nematode muscle receptors offers new opportunities for *in vitro* screening of new candidate cholinergic anthelmintic drugs.

Note that the full genomic sequence of *C. elegans* does not identify the *unc-63* gene cDNA sequence as such, and there is no information in the genomic sequence to indicate that a cDNA sequence from *C. elegans* could permit function recombinant expression of a major anthelmintic drug target (namely the nematode nicotinic acetylcholine receptor) which would effectively mimic the natural receptor.

Figure Legends

Figure 1. Deduced amino acid sequence of the JTF-38 nAChR α subunit of *C. elegans*.

amino acids are numbered beginning at the first methionine. Loops contributing to the ACh binding domain are underlined by plain line. The bilayer spanning

transmembrane regions TM1-TM4 are underlined by broken lines. The horizontal arrow with a broken line indicates the intrachain di-sulfide bond. The two adjacent cysteines, typical of all nAChR α-like subunits are shown in bold [SEQ ID No. 1].

Figure 2. Chromosomal localization of the *C. elegans jtf-38* gene
Genetic map position of *unc-63* on chromosome I. The YACs Y55F5, Y72D6 and Y72E2 span the *unc-63* locus.

Figure 3. Genomic organization of the *jtf-38* gene.
The genomic organization of jtf-38 gene is depicted. Boxes indicate exons. SL1 refers to the site of attachment of the *trans*-splice leader SL1. Three subclones, punc-63.1, punc-63.2 and punc-63.3 of the *jtf-38* gene have been tested for their ability to rescue the normal wild type phenotype in *unc-63* mutants.

Figure 4. Dendogram showing UNC-63 and related nAChR subunit family members.
(A) shows *C. elegans* in relation to other known nicotinic receptor subunits. (B) shows related subunits with *C. elegans.*

Figure 5. Amino acid sequence comparison of UNC-63, UNC-38, LEV-1 and UNC-29.

Figure 6. Functional expression in *Xenopus laevis* oocytes of UNC-63, UNC-29 and LEV-1 results in a functional nAChR at which ACh is an agonist whereas levamisole and nicotine are partial agonists. The agonist action of levamisole is blocked by 10 μM mecamylamine.

Materials and methods

C. elegans strains and general methods

Worm culture, handling followed the technique described by Sulston and Hodgkin (1988). The wild type *C. elegans* was the Bristol N2 strain (Brenner, 1974). Strains containing unc-63 alleles zz37, zz26, b404 were obtained from the *Caenorhabditis* Genetic Center (University of Minessota).

Sequence analysis

Sequence alignment and analysis were performed with the GCG packaging, CLUSTALW and BLAST.

Molecular biology

Methods published by Sambrook et al.(1989) were used unless otherwise stated. *C. elegans* genomic was prepared from wild type as described by Koelle. Plasmid DNA was prepared using Tip 100 from Qiagen. Sequencing was performed according to Sanger et al. with fluorescent dye terminator for automated sequencing.

Cloning of jtf-38 cDNA

A mixed stage cDNA library in λgt10 was screened with a probe for *unc-38* (Fleming et al., 1997) at moderate stringency 65°C in 2xSSC, 0.1%SDS. One of the positive clones JTF38 was subjected to further analysis. Sequencing of a 0.6kb EcoRI fragment suggested that the clone contained a genomic insert encoding a nAChR subunit.

An antisense oligonucleotide HAB085 was designed which recognized the putative coding sequence from this clone. This oligonucleotide was used in a 5' RACE reaction using Marathon cDNA (Clontech) derived from total RNA for mixed stage N2 *C. elegans*. The reaction was carried out using TaqPlus DNA polymerase mix (Stratagene). A band of c. 900bp was cloned. Sequencing of the ends demonstrated a sequence identical to the JTF38 clone at the 3' end and a possible nAChR mRNA 5' end at the other.

An oligonucleotide HAB112 to the 5' end was then used to generate a full length cDNA clone by 3' RACE on Marathon cDNA using Expand polymerase mix (Boehringer Mannheim). A band of 1.6kb was isolated and cloned into pGEM-T (Promega) to yield the plasmid pHAB385.This insert in this clone was sequenced. The fragment was then excised as a *Not*I fragment, using sites in the pGEM-T polylinker and in the Marathon cDNA adaptor and ligated into pMT-3. The orientation of clones was established by PCR and the vector insert junction confirmed by DNA sequencing. A clone of the appropriate structure was named pHAB386.

Genetic localization of jtf-38 gene

The jtf-38 cDNA was used to probe an ordered grid of yeast artificial chromosome (YAC) clones representing most of the *C. elegans* genome. The jtf-38 cDNA was labeled by random priming with 50 μCi of ^{32}P dCTP. Hybridization has been done following the protocol described by Coulson et al. (1995) with 10^6 cpm/ml of hybridization buffer.

Mutation detection

Total RNA was isolated using Trizol reagent (Life Technologies) from three different *unc-63* allele mutant populations. 2 μg of total RNA was used in each reverse transcription with pdN6 primers, following the manufacturer instructions (Expand reverse transcriptase kit, Boerhinger). Subsequently 5μl of RT product was used in PCR. We used 4 pairs of primers, deduced from the jtf cDNA sequence, and Taq polymerase from Promega. PCR experiments were run for 40 cycles (denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min). PCR products were cloned into pGEM-T (Promega) and sequenced. Two different clones were sequenced at least for each product. When the mutation was determined the corresponding genomic was sequenced to confirm it. In this case single worm PCR was performed. Briefly 5 mutant worms were picked from the plate and transferred into a lysis buffer containing 1X expand buffer from

Boeringher and proteinase K (0.05 mg/ml). Worms were denatured by 1 hour incubation at 65°C following by 10 min incubation at 95 °C. Subsequently 5 µl of lysis worms are used for a PCR using primers deduced from the genomic region showing the mutation.

Germline transformation

Germ line transformation was performed according to the method of Mello and Fire (1995).

Mutant rescue experiment:

For rescue experiments 3 different constructs, p*unc-63*.1, p*unc-63*.2 and p*unc-63*.3 were generated by means of PCR using the Expand Long Template System (Boeringher) on genomic DNA isolated from N2 worms as described by Koelle (1988). PCR products were cloned into the pGEM-T plasmid. Clones were subsequently checked for the presence of known restriction sites and by sequencing both 5' and 3' extremities. The p*unc-63*.1 construct contains a 12.5kb insert comprising 4.5kb of 5' region, the all genomic coding region and 1kb of 3' untranslated sequence p*unc-63*.2 contains a 10kb insert and differs from p*unc-63*.1 in that it has only 2.5kb of genomic sequence upstream of the *unc-63* ATG site. The p*unc-63*.3 construct differs from p*unc-63*.2 only by 0.7 kb of 3' untranslated sequence. Germ line transformation was performed by co-injecting the test DNA at a concentration of 100-120 ng ml^{-1} and the plasmid pPD93 65 which contains the GFP gene under the control of the promoter for *unc-54*, the myosin heavy chain gene expressed in all muscle cells. Transgenic animals are therefore selected by GFP fluorescence in body wall muscle cells and grown on individual plates enabling studies on the phenotype of rescued worms (levamisole sensitivity, normal locomotion and egg laying).

GFP localisation:

A 10.6 kb fragment was amplified from genomic DNA by means of PCR, using the Expand Long Template system (Boeringher), with primers designed to contain SphI (sense primer) and XmaI (forward primer) restriction sites at one end. The fragment includes 4.5 kb of the putative 5' regulatory region and 6kb of genomic coding sequence including exon I through part of exon 7 (encoding the TM3-TM4 extracellular loop). This fragment has been cloned in frame into the GFP expressing vector pPD95.70 using the engineered restriction site at both ends of primers. This construction has been designated UNC-63::GFP1.

The fusion construct UNC-63::GFP1 at a concentration of 80 mg ml^{-1} was co-injected with plasmid pRF4 (100ng μl^{-1}) into wild type animals. Transgenic animals were selected by their roller phenotype and viewed by fluorescence microscopy (Zeiss Axiovert 35 filtersX).

Functional expression in *Xenopus laevis* oocytes

Ovaries were surgically removed from anaesthetised mature female *Xenopus laevis*. The follicle layers were manually removed from healthy stage V and VI oocytes following a 15 min incubation with collagenase (type IA, 2 mg ml^{-1}) in a calcium-free version of standard oocyte saline. The composition of SOS was as follows (mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5; pH 7.6. In calcium-free saline, the $CaCl_2$ was replaced by 1.8 mM $BaCl_2$. The UNC-63-pMT_3, UNC-29-pMT3, LEV-1-pMT_3 and UNC-38-pMT_3 expression constructs (Swick *et al.*, 1992) were isolated from *E.coli* JM109 using endo-toxin free maxi-prep kits (Qiagen). The nucleus of each oocyte was injected with 20 nl of DNA (0.1 $\mu g\ \mu l^{-1}$). The injected oocytes were transferred to incubation medium composed of SOS supplemented with penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$), gentamycin (50 $\mu g\ ml^{-1}$) and 2.5 mM sodium pyruvate and placed at 4°C for 30 min immediately after injection to enable recovery. Oocytes were maintained at 16°C for 2-4 days prior to electrophysiological studies.

Electrophysiology

Oocytes were restrained with entomological pins in a Perspex chamber (80 µl volume) with a Sylgard base and perfused continuously (5 ml min^{-1}) with SOS using a gravity- fed system. Membrane currents were measured by the two-electrode voltage-clamp method, using 3M KCl filled electrodes (resistance = 0.5 - 5 MΩ) and either a Geneclamp amplifier (Axon Instruments). The oocyte membrane potential was clamped at -100 mV. Signals were digitized by a TL-1 interface (Axon Instruments, U.S.A).

Chemicals

Acetylcholine chloride, nicotine, levamisole and mecamylamine were obtained from Sigma. Unless otherwise indicated all other chemicals were obtained from Sigma (UK).

Results

Cloning a novel *C. elegans* nAChR subunit

A *C. elegans* cDNA phage (λ7) library was screened at low stringency using the *unc-38* and *unc-29* cDNAs as probes. Several positive clones were obtained. One positive clone, jtf-38, hybridized specifically at low stringency with *unc-38* and was investigated further. This positive clone was a partial cDNA but showed 50 % identity at the amino acid level with UNC-38. We then utilised 5'RACE to identify the 5' end of the mRNA and showed that the transcript was transpliced at its 5' end to the RNA splice leader SL1 (Krause and Hirsh, 1987). The SL1 sequence was found upstream the putative methionine ATG. We then used 3'RACE to amplify a full length cDNA clone. This cDNA was designed jtf-38.

The jtf-38 clone encodes a nicotinic acetylcholine receptor subunit

Sequence analysis of clone jtf-38 revealed an open reading frame of 502 amino acids. The presumptive deduced protein has a calculated molecular weight of 65 kDa. The sequence shows all the characteristics (cf Galzi and Changeux 1995, Hucho et al 1996) of a new nAChR α subunit. We found conserved stretches of amino acids involved in the ACh binding site including loop A, loop B and loop C motifs as well as the 'cys loop' defined by the di-sulfide bridge between cysteines 151 and 165. The two adjacent cysteines, typical of α subunits, are located at amino acid positions 241 and 242. In contrast to UNC-38 (Fleming et al., 1997) and several other *C. elegans* nicotinic receptor (Mongan et al., 1999), the JTF-38 subunit had the typical Y-X-C-C motif in the putative loop C of the ACh binding site.

There are 4 putative transmembrane domains, TM1 (260-283), TM2 (291-308), TM3 (324-344) and TM4 (464-476). The GCG MOTIFS program identified 3 putative phosphorylation sites all included into the intracellular loop TM3-TM4. It has been shown that the *Torpedo* receptor can be phosphorylated by at least three different protein kinases: cAMP - dependant kinase (PKA), protein kinase C (PKC) and a tyrosine kinase (TK). The putative sites on *unc-63* are of the PKC (2) and TK (1) type.

Physical and genetic locations of the *C. elegans* gene identified by the jtf-38 clone

We mapped the physical location of this new nAChR gene to YACs Y55F5 and Y72D6, in a cosmid gap, by hybridizing the cloned cDNA to the YAC grid. Thus the jtf-38 gene maps to the center of chromosome I. In this genomic region defined by these two overlapping YACs, lies the levamisole resistant loci *unc-63*. On the other hand previous work has shown that a small YAC Y72E2, which overlaps partially Y55F5 and completely Y72D6, has been injected in the mutant *unc-63* and rescued the levamisole resistance conferring *unc-63* mutant (T. Barnes, personal communication). We therefore tested whether JTF-38 was *unc-63*. We thus determined the entire coding sequence of the corresponding JTF-38 cDNA in three different *unc-63* mutant alleles.

Several classes of *unc-63* mutations

We generated jtf-38 cDNA by RT-PCR from three different mutant alleles of *unc-63*. The *x37* mutant allele worms are inactive, slow and extremely resistant to levamisole. For this allele we found a single base transition G:C to A:T at the flanking region of intron 4. The intron 4 is not correctly *cis*-spliced, changing the open reading frame and introducing a stop codon in-frame.

The *b404* mutant allele has a slight levamisole resistance and a slight uncoordinated movement. We found a deletion (138 nt) in the coding sequence of the M3-M4 intracellular loop. This deletion kept in phase the two remaining fragments. This deletion removes conserved amino acids and at least the three putative phosphorylation sites. Moreover it has been recently showed, *in vivo*, that this large intracellular loop is also involved in the localization of nAChR to the active site at the synapse (Williams et al., 1998).

The *x26* mutant allele has normal movement and slight levamisole resistance. We found a G:C to A:T transition changing the cysteine 151 to a Tyrosine. This cysteine part of the loop B involves in the ACh binding site. This same mutation has been shown recently to be involved in a human congenital myasthenia syndrome (Milone et al 1998). The mutant receptor subunit fails to incorporate into the cell surface and is therefore a null mutation.

Thus the findings for the *unc-63* mutant alleles provide evidence that the nAChR ∀ subunit cDNA we cloned and *unc-63* are the same gene. In the course of this study the complete genomic sequence of YAC 72E2 covering the *unc-63* locus was determined by the Sanger Center. We therefore compared the *unc-63* genomic and cDNA sequences. The *unc-63* gene is composed of 10 exons spanning 7.5 kb. To examine the expression of *unc-63* we performed a Northern blot hybridization and detected a single transcript.

***unc-63* is expressed in both muscle cells and neurons**

To address the question of the localization of *unc-63* we monitored its expression by fusing to GFP (Green Fluorescent Protein) (Chalfie et al., 1994) a genomic region comprising 4.5kb of 5' upstream, promoter - containing sequence and the genomic *unc-63* coding region encompassing the first 7 exons. In transgenic animals expressing the UNC-63::GFP construct, fluorescent signals were observed in all body wall muscle cells and in vulval muscle cells. We also found expression in many cells of the nervous system, including motor neurons. These findings are consistent with the *unc-63* mutant defects as mutant worms have defects in locomotion and exhibit abnormal egg laying rate. This expression pattern suggests that *unc-63* functions in both muscle and nerve cells of *C. elegans*.

The *C. briggsae* genome contains a very close relative of the *C. elegans unc-63* gene.

The divergence between the two closely related nematode *Caenorhabditis elegans* and *Caenorhabditis briggsae* is as large as between mammals and reptiles (Fitch et al., 1995). Many proteins have both a very high level of conservation in sequence (Grauso et al. 1996) and in function between the two species (Kennedy et al., 1993; Krause et al., 1994). Using PCR and primers deduced from the *C. elegans unc-63* sequence we amplified a nearly full length cDNA. The high homology (95%) between the 2 sequences strongly suggests that we have cloned the *C. briggsae unc-63* homologue.

Functional expression of *unc-63* in *Xenopus* oocytes

When cDNAs encoding *unc-63*, *lev-1* and *unc-29* were injected separately into *Xenopus laevis* oocytes no evidence of ACh - induced currents was obtained. Pairwise injections of all combinations were similarly unsuccessful in generating ACh - induced currents. However, when all 3 cDNAs were co – injected robust ACh – induced currents (inwardly directed) were detected at a holding potential (E_h)

of –100mV. The ACh dose - response curve resulted in EC_{50} value of 20μM. Whereas ACh was a full agonist, levamisole and nicotine showed partial agonist activity on the expressed heterotrimeric UNC-63, UNC-29, LEV-1 receptor. Mecamylamine (10μM) was an effective antagonist of the ACh – induced currents 5recorde from the expressed UNC-63, UNC-29, LEV-1 heterotrimeric receptor. Thus this robust recombinant heteromeric nAChR resembles in respect of ACh, nicotine and levamisole and mecamylamine actions the native muscle nAChR of *Ascaris suum* muscle (Colquhoun *et al.,* 1991; 1993).

Discussion

UNC-63 is a new *C. elegans* nicotinic acetylcholine receptor (nAChR) α subunit. Its amino acid sequence shows the vicinal cysteine motif by which such subunits are defined. There are 4 putative transmembrane subunits, a long N-terminal region containing sites that show strong conservation with the loops (A-F) which appear to make up ACh binding site (Lena and Changeux 1998). In the case of UNC-63 the vicinal cysteines are part of a Y-X-C-C motif in loop C. Based on its amino acid sequence homology UNC-63 is designated a member of the UNC-38 - like nAChR subunit family, all members of which identified to date are ∀ subunits. Using a gfp fusion construct we have shown that UNC-63 is expressed in all body wall muscles, in vulval muscles and in certain motor neurons of *C. elegans*. UNC-63 and UNC-29 are both strongly expressed in body wall muscle of *C. elegans* (as well as in certain neurons) but nothing is known to date of the expression of LEV-1.This pattern of spatial distribution with a nAChR subunit not being confined to a particular cell or tissue type has now been found for several *C. elegans* nAChR subunits (see Table 1), a situation that contrasts strikingly with the situation that so far obtains in vertebrates where separate gene families are expressed in nerve and muscle.

UNC-63 when co-expressed in *Xenopus* oocytes with UNC-29 and LEV-1 results in the most robust functional expression observed to date for a *C. elegans* recombinant heteromeric nAChR and indeed for any recombinant heteromeric

nAChR containing only invertebrate subunits. For example, earlier work on *C.elegans* recombinant nAChRs using the UNC-38, UNC-29 and LEV-1 combination did result in functional heteromeric receptor but the current amplitudes were much lower than those reported here. Interestingly when we co-expressed UNC-38 with the the other 3 subunits used throughout the present study, this reduced the amplitude of the currents recorded. It is not clear why UNC-38 has this effect. It does have an unusual Y-X-X-C-C motif in loop C of the ACh binding site which may conceivably impair normal ACh - receptor interactions but other factors also remain to be investigated. Certain neuronal nAChR receptors of vertebrates are known to contain 2 distinct ∀ subunits notably ∀3 and ∀5. The ∀5 subunit also has an atypical (A-X-C-C) loop C motif. It may be that UNC-63 and UNC-38 are not normally expressed in the same nAChR molecule. The possibility that another non-∀ subunit is required for UNC-38 to exert its full functional role cannot be discounted. A possible functional role may exist for a 'silencing subunit' eg early in development or at the dauer stage. Changeux and colleagues have suggested a labelling role for nAChRs in synapse formation early in development. Alternatively, this effect may simply be the result of mis-assembly of subunits that don't normally belong together.

Thus of the 11 genes linked to levamisole resistance 4 are now known to be nAChR subunits and one other remains a possible candidate.

The gene *unc-63* encoding the new ∀ subunit described here is located on chromosome I of *C. elegans*. The 2 other known members of the UNC-38 - like group of ∀ subunits are located on the same chromosome. None are in sufficiently close proximity to be part of a common transcription unit as is the case for other ∀ subunit genes such as (*deg-3, des-2*).

Mutants of *unc-63* have proved to be instructive. The mis-sense mutation in allele *x37* which results in the insertion of a stop codon gives rise to a phenotype showing very strong resistance to levamisole and slow movement. In the *x26* mutant allele, which appears to have normal movement and only slight levamisole resistance, the cysteine at position 151 is replaced by a tyrosine. The effect of this

change in the N-terminal region (part of loop B) is to open up the cys loop, an effect similar to that produced by one of the ∀ subunit mutations resulting in a congenital myasthenic syndrome.

Conclusion

This new ∀ subunit has permitted the first robust functional expression of a nematode nAChR on which levamisole has similar actions to those observed on native nematode muscle nAChRs. This transient expression system and in future a stable cell line containing such recombinant receptors offers the prospect for the first time of rapid high - throughput screening for a new generation of cholinergic anthelmintics and endectocides.

Table 1

Subunit	Location of expression	Method	References
deg-3, des-2	PVC, PVD, FLP (touch neurons) IL2 neuron head muscle cell	LAC-Z reporter gene	Treinin and Chalfie, 1995 Treinin *et al.*, 1999
unc-29	Body wall muscle cells head neurons	GFP reporter gene	Fleming *et al.*, 1997
acr-5	B-type motor neurons	GFP reporter gene	J. Ahringer, pers. comm..
acr-2	Motor neurons (multiple classes)	GFP reporter gene	H.R. Horwitz, pers. comm.
unc-38	Muscle cells and neurons	Genetic interactions of *unc-38* with both *unc-29* and *acr-2* mutants	H.R. Horwitz, pers. comm.
unc-63	Body wall muscle and Motor neurons (multiple classes)	GFP reporter gene	E. Culetto, pers. comm.

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